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Original Article

BIOTECHNOLOGICAL PRODUCTION OF α-AMYLASE THROUGH SUBMERGED FERMENTATION BY *BACILLUS MEGATERIUM* KLMI4 USING AGRO-WASTES AS SUBSTRATE

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ABSTRACT

Different agro-wastes (rice husk, groundnut oil cake and green gram husk) with different quantities (1, 3, 5, 7 and 10 g) were inoculated with 1 ml of bacterial suspension and incubated at 37 ^{0}C (room temperature) in rotary shakers. At every 24 h intervals samples were withdrawn from each flask and amylase production was estimated. The highest yield of 9 IU was recorded with 5 g concentration in case of rice husk at 48. Similarly 10 IU and 42 IU was recorded with 5 g concentration at 48 h in both the cases of Groundnut oil cake and Green gram husk respectively. Total carbohydrate, total protein, and total fats were estimated of three agro-wastes. Basal parameters (pH, Temperature, and Inoculum size) were optimized. Enzyme activity increased up 75 IU at pH 8.0, 125 IU at 37^0 and at 3.0x10^{6}cfu/ml, the highest enzyme activity of 128 IU was observed after 48 h incubation.

KEYWORDS*: *Bacillus Megaterium KLMI 4, Alpha-Amylase Production, Agro-Wastes (Substrate) & Optimization of Basic Parameters

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INTRODUCTION

Different agro-wastes have been employed world-wide to as substrates to produce α-amylase through microbial fermentations (either SmF or SSF) and every now and then newer agro-wastes are added to this list. The main purpose of using agro-wastes in place of well-defined components is primarily to reduce costs involved in production of the enzyme utilizing the carbohydrate content of these wastes and as a by-product, there is an abatement of environmental pollution. Most commonly used agro-wastes are the rice husk, brans of wheat, maize, corn, millets and rice, oil cakes of cotton seeds, coconut, groundnut, sunflower, palm, soya, etc., sugarcane bagasse, cassava peels, fruit peels, gram wastes, spent brewing grains, and even kitchen wastes.

MATERIALS AND METHODS

Preparation of the Agro-Wastes for Fermentation

Different locally available agro-wastes like groundnut oil cake, green gram husk and rice huskwere obtained and employed to evaluate whether these could support α-amylase production by the bacterium in concern through submerged fermentation. The rice husk, groundnut oil cake, and green gram husk procured from the local market. Each was thoroughly cleaned to remove any foreign impurities, finely ground and sieved through 85 mesh standard sieves and fine powder was once again ground in a mixer to obtain a finer powder. The green gram was

purchased in the local market; foreign materials were removed and the grains were dipped in water for 30 min to loosen the husk. Then the moistened grains were heat-dried overnight at a temperature of 60^0 C in a hot air oven. It was de-husked next day in a grinder. Approximately 150 to 250 g husk was obtained from one kg of whole grains. Required quantities of each agro-waste was finely ground and sieved through 85 mesh standard sieve to remove any coarse particulates. The sieved husks were individually ground once again in a mixer to obtain fine powders.

Composition of Agro-Wastes

The finely ground agro-wastes were individually assessed for their gross organic contents (carbohydrate, protein and fats).

Estimation of Total Carbohydrates

The total carbohydrate content of the different agro-wastes was estimated as per the method of Dubois *et al* (1956).

Estimation of Total Protein

Total protein content of the different agro-wastes was determined by colorimetric method of Lowry *et al* (1951).

Estimation of Total Fats

Total fat content of the agro-wastes was estimated through the Soxhlet method by extracting with petroleum ether.

Preparation of the Fermentation Medium

Required quantities of each agro-waste were separately dispensed into beakers containing 100 ml of distilled water and boiled for 10 min. The suspension was cooled and filtered through What man filter paper no. 1. The filtered extract volume was made up to 100 ml with distilled water and 0.5% NaCl was added. This served as the fermentation medium to evaluate α-amylase production by the bacterial strain.

Optimization of the Process Parameters using Agro-Wastes as Substrate

Extracts of different quantities (1, 3, 5, 7 and 10 g) of each of the three agro-wastes were prepared as detailed above, amended with 0.5% NaCl and made up to 100 ml were dispensed separately in 250 ml Ehrlenmeyer flasks and autoclave sterilized at 121^0 C for 15 min and cooled to room temperature. Thereafter the extract was aseptically inoculated with a bacterial suspension ($3x10^6$ CFU/ml) and incubated in a rotary shaker incubator at 200 rpm and 37^0 C over a length of time. Required quantities of samples were withdrawn every 24 h and analyzed for α-amylase activity as detailed earlier. Studies were conducted in triplicate.

- **Optimization of *p*H**

The *p*H of the green gram husk extract before autoclave sterilizing was adjusted to different levels using 0.1N NaOH and 0.1 HCl. Studies were conducted in the *pH range* of 5.0 to 10.0 (with intervals of 0.5). After initial *p*H levels adjustments, the individual flasks were each inoculated with a bacterial suspension ($3x10^6$ CFU/ml) and incubated at 37^0 C in a rotary shaker incubator. Samples were withdrawn from each flask for estimating α-amylase activity at regular intervals of 24 h. Study was conducted in triplicate.

- **Optimization of Temperature**

In order to evaluate the influence of temperature, the flasks with fermentation medium inoculated with the bacterial suspension and optimum initial *p*H adjustment were incubated at different temperature levels in rotary shaker incubators. Studies were carried out at temperature levels of 21 to 49^0 C with intervals of 2^0 C. Samples were withdrawn from each flask for estimating α-amylase activity at regular intervals of 24 h. A study was conducted in triplicates.

- **Optimization of Initial Inoculum Size**

The seed inoculum was prepared as earlier and it was diluted, if necessary, to yield $3x10^6$ CFU/ml of bacterial suspension. Different volumes of inocula ($1x10^6$cfu/ml, $2x10^6$cfu/ml, $3x10^6$cfu/ml, $4.5x10^6$cfu/ml and 6.0 $x10^6$cfu/ml) were dispensed aseptically into the experimental flasks containing green gram husk to determine the optimum level of inoculum size to effect maximum α-amylase production. The flasks were incubated under optimum *p*H and temperature conditions established earlier. Samples were withdrawn from each flask to estimate α-amylase activity at 24 h intervals. The study was conducted in triplicates.

RESULTS

Attempts have been made to evaluate the feasibility of three different agro-wastes (rice husk, groundnut oil cake, and green gram husk) as substrates for α-amylase production by the present strain, *B. megaterium*KLMI4, through submerged fermentation.

- **Biochemical Evaluation of the Agro-Wastes**

Table 1: The Composition of the Five Agro-Wastes (Carbohydrates, Proteins and Fats) are Presented in Table

	Rice Husk	Groundnut Oil Cake	Green Gram Husk
Total carbohydrates	25.2%	27.05%	60.45%
Total proteins	16.2%	48.75%	7.62%
Total fats	22.90%	2.2%	2.15%

- **Optimization of Process Parameters of Submerged Fermentation**

The optimization of the basic process parameters (*p*H, temperature, inoculum size and substrate concentration) is very important in any submerged fermentation study. This was done with the basal medium used earlier. While employing agro-wastes as substrates, the process parameters need to be standardized. Moreover, since it has been aimed to select the agro-waste that can support high α-amylase production, it becomes apparent to attempt initially to optimize substrate concentration as a first step which also would indicate the most suitable one amongst the five agro-wastes for production of α-amylase.

Optimization of Substrate Concentration and Selection of the Substrate

The extract prepared from each agro-waste (boiling separately 1, 3, 5, 7 and 10 g/100 ml agro-waste in 100 ml distilled water, cooling and making up the volume to 100 ml and supplementing the extract with 0.5% NaCl) served as the fermentation medium. Each of the agro-waste extract formulation was separately inoculated with 1 ml bacterial suspension (10^6cfu/ml) and incubated at room temperature of 30 ± 0.5^0 C for a period of 96 h.

- **Rice Husk Extract**

The results obtained from the studies involving submerged fermentation of rice husk extract are presented in Figure 1

Enzyme production in all the concentrations exhibited a similar trend, i.e., yield rose up to the 48 h incubation wherein maximum values were observed and thereafter it decreased sharply up to the 96 h incubation period. The highest yield of 9 IU/ml was recorded with 5 g concentration at 48 h.

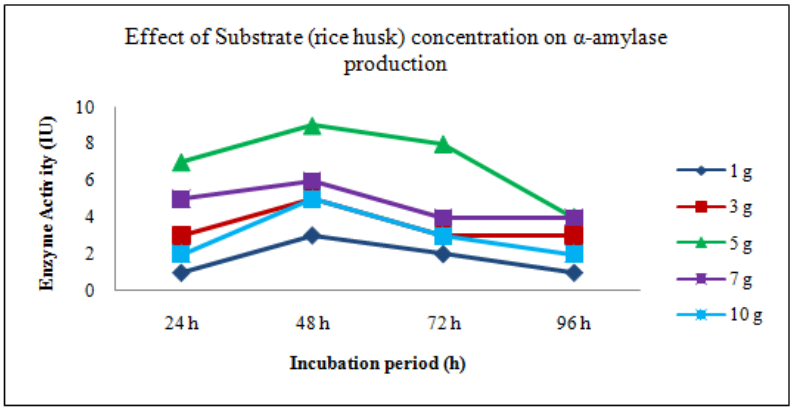

Figure 1: Effect of Substrate (Rice Husk) Concentration on α-Amylase Production

- **Groundnut Oil Cake Extract**

The results obtained from the studies involving submerged fermentation of groundnut oil cake extract are presented in Figure 2.

Enzyme production in all the concentrations exhibited a similar trend as with rice husk extract, i.e., yield rose up to the 48 h incubation wherein maximum values were observed and thereafter it decreased sharply up to the 96 h incubation period. The highest yield of 10 IU/ml was recorded with 5 g concentration at 48 h.

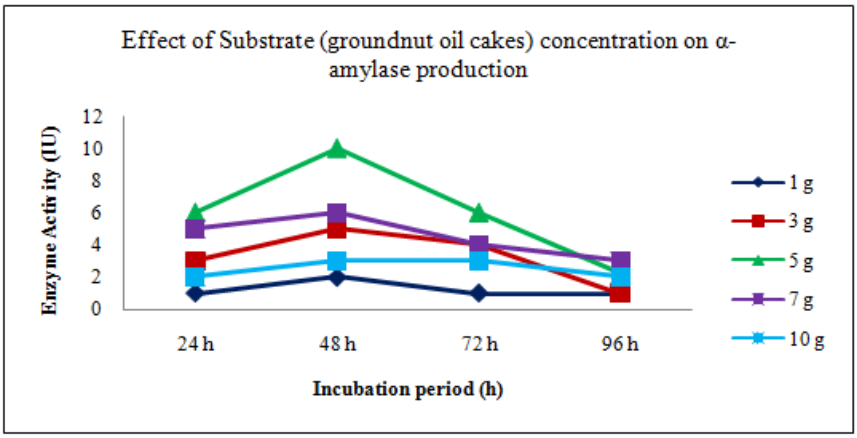

Figure 2: Effect of Substrate (Groundnut Oil Cakes) Concentration on α-Amylase Production

- **Green Gram Husk Extract**

The results obtained from the studies involving submerged fermentation of green gram extract are presented in Figure 3

Though the enzyme production in all the concentrations exhibited a similar trend as with other ext

racts, the yields recorded were higher. Enzyme yield rose up to the 48 h incubation wherein maximum values were observed and thereafter it decreased sharply upto the 96 h incubation period. The highest yield of 42 IU/ml was recorded with 5 g concentration at 48 h.

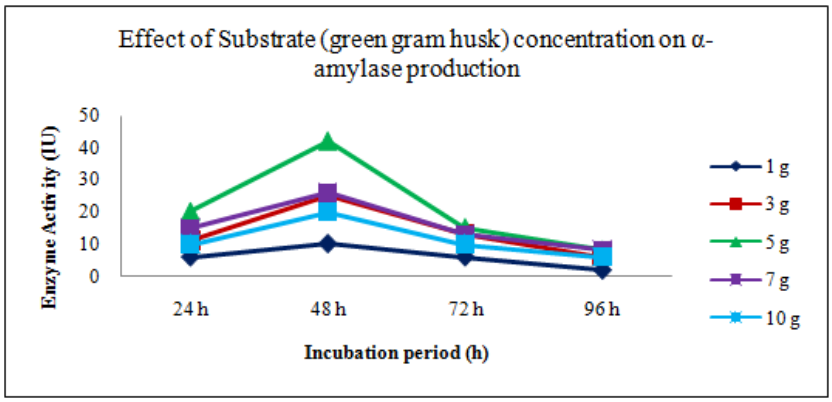

Figure 3: Effect of Substrate (Green Gram Husk) Concentration on α-Amylase Production

Selection of the Substrate with Maximum Potential for α-Amylase Production

This study indicates that 5 g extract of any of the agro-wastes is essential for the optimum production of α-amylase. An evaluation of the α-amylase production by *B. megaterium* KLMI4 through submerged fermentation after 48 h of incubation indicates that yield by the different agro-wastes is in the order of green gram husk>groundnut cake>rice husk. Thus amongst all the agro-wastes studied, the extract of the green gram husk possesses the best potential for α-amylase production by *B. megaterium*KLMI4 as is deduced from Figure 3.

Optimization of *p*H

These studies were conducted for a period of 96 h in the *p*H range of 5.0 to 10.0, with intervals of *p*H 0.5. The results are presented in Figure 4.

In the *p*H levels of 5.0 and 5.5, enzyme activity was not observed in the first 24 h; after 48 h and 72 h incubation, very low activity of 2.0 and 1.0 IU/ml, respectively, was recorded and once again nil activity was discernible after 96 h. But as *p*H level increased, enzyme activity increased up to *p*H 8.0, wherein maximum enzyme activity (75 IU/ml) was recorded at 48 h incubation. In higher *p*H levels (from *p*H 8.5 onwards) it decreased considerably; at *p*H 10.0, a low enzyme activity (10 IU/ml) only at 48 h incubation and nil activity all other incubation periods were recorded. The enzyme retained more than 50% of its activity at *p*H levels of 7.0 and 9.0 after 48 h incubation (Figure 4). Generally, the enzyme activity after different incubation periods exhibited the trend of 48 h>24 h>72 h >96 h.

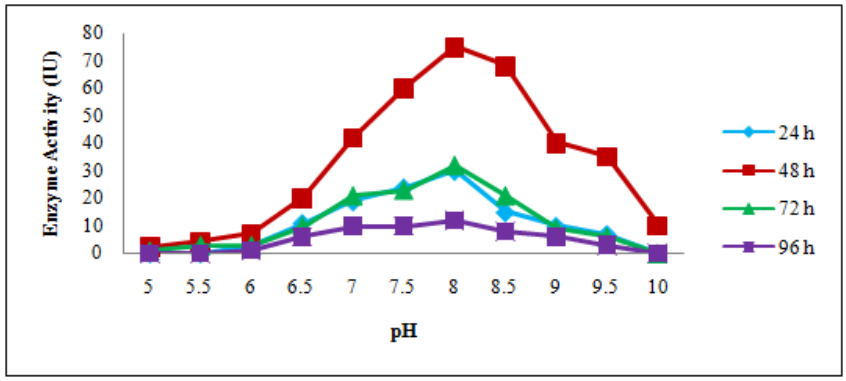

Figure 4: Effect of pH on α-Amylase Production

Optimization of Temperature

The range of temperature in which these studies were carried out is from 21^0 C to 49^0 C, with intervals of 2^0 C. The *p*H of the fermenting medium was maintained at 8.0.The results are presented in Figure 5.

Starting with low enzyme activity at 24 h, it sharply rose to the peak at 48 h and thereafter it declined sharply at 72 and 96 h, which was the general trend observed at all the temperature level. Very low enzyme activity was recorded at 21^0 and 23^0 C during the fermentation period of 96 h. The enzyme activity increased gradually from 25^0 C to reach maximum (125 IU/ml) at 37^0 C at 48 h. It started to decline sharply from 39^0 C, once again reaching very low levels at 49^0 C. In general, the relative enzyme activity was above 30% at 29^0 C and 45^0 C after 48 h of incubation (Figure 5). The enzyme activity after different incubation periods exhibited the trend of 48 h>24 h>72 h >96 h.

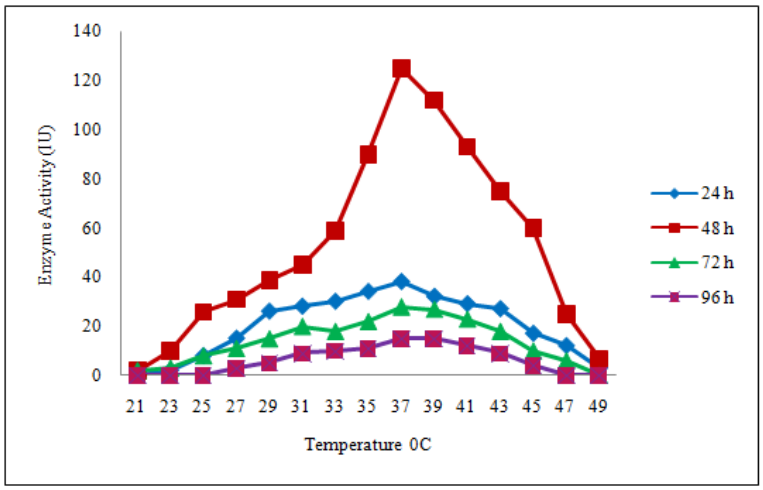

Figure 5: Effect of Temperature on α-Amylase Production

Optimization of Inoculum Size

These studies were conducted employing different inoculum sizes: 1.0x, 2.0x, 3.0x, 4.5x and $6.0x10^6$cfu/ml. The *p*H and temperature of the fermenting medium was maintained at 8.0 and 37^0 C, respectively. The results are presented in Figure 6.

It has been generally observed that the enzyme activity was low at inoculum of $1.0x10^6$ cfu/ml and it increased concurrently with the increase in inoculum up to $3.0x10^6$cfu/ml at all incubation periods. At $3.0x10^6$cfu/ml, the high

enzyme activity of 128 IU/ml was observed after 48 h incubation. With further increases in inoculum size, the enzyme activity decreased. However, almost 60% of the activity was retained with 6. $0x10^6$cfu/ml inoculum. The enzyme activity at 48 h incubation period exhibited the following trend: 3. $0x10^6$cfu/ml >$4.5x10^6$cfu/ml >2. $0x10^6$cfu/ml and 6. $0x10^6$cfu/ml>1. $0x10^6$cfu/ml.

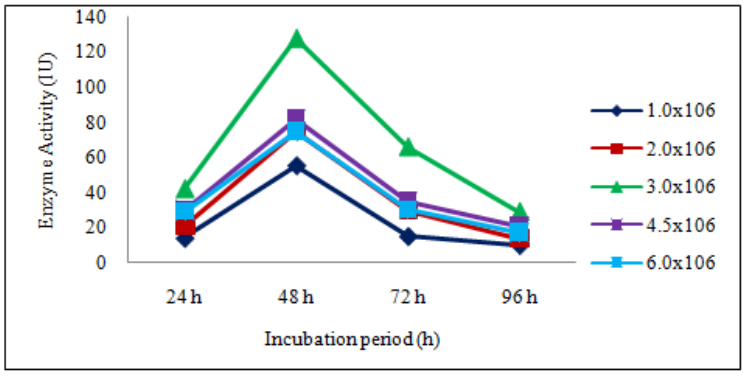

Figure 6: Effect of Inoculum Size on α-Amylase Production

DISCUSSION

Studies pertaining to use of green gram husks as a source of C for α-amylase production are limited. Chimata (2010) reported green gram husk to be less beneficial than brans of wheat and rice and observed that its use as a C source led to 73 U/gds of α-amylase yield with *Aspergillus*sp. in SSF condition. Sharada (2012) reported green gram husk to be a better supporter of α-amylase production than the black gram husk. It is very difficult to compare U/gds with IU/ml. However, it can be safely assumed that green gram husk can serve as a good source of C for either SSF or SmF.

The optimization of basic process variables (*p*H, temperature, inoculum size and substrate concentration) using the agro-wastes did not vary from the observations made using the basal medium. The only difference is the of use of a cheaper agro-waste amended with some common salt, yet producing higher α-amylase, when compared to that obtained from the basal medium made up of relatively costly chemical compounds. When all the process variables were optimized, the relatively much cheaper medium of green gram husk yielded higher enzyme yield of 128 IU/ml compared to 93 IU/ml production obtained with costlier basal medium.

Different agro-wastes have been successfully used for this purpose through SSF and SmF processes as a result of their complex organic composition (carbohydrates, proteins, fats, etc.) and an attempt has been made to list them in the Table.

Table 2: Use of Different Agro-Wastes for Microbial α-Amylase Production

Microorganism	Substrate/Method of Fermentation	Reference
Aspergillusoryzae NRRL 1808	Spent brewing grains (SSF)	Bogar*et al.* (2002)
Bacillus amyloliquefaciens	Wheat bran, groundnut oil cake (SSF)	Gangadharan*et al.* (2006)
A.oryzae	Wheat bran, rice husk, cassava bagasse, seed powders of jack fruit & tamarind, palm kernel cake, & oil cakes of coconut, ground nut, seasame, olive, mustard & cotton seed. (SSF)	Sivaramakrishan*et al.* (2007).
*Aspergillus*sp.	Wheat and rice brans, green gram husk.(SSF)	Chimata*et al.* (2010)

Bacillus sp.	Brans of wheat & rice, green gram husk, mustard oil seed cake. (SSF)	Saxena and Singh (2011)
A. niger	Wheat bran, rice husk, wastes of potato, tomato, brinjal& banana peel (SSF)	Khan *et al.* (2011)
Tricheciumroseum	Wheat bran, rye staw, corncob leaf, sunflower oil meal, rice husk. (SSF)	Balkan *et al.* (2011).
Bacillus sp.	Cassava (SSF)	Senthilkumar*et al.* (2011).
A. niger	Potato peel (SSF)	Mahmood*et al.* (2016).
*Naxibacter*sp.	Sugarcane bagasse, potato peel, kitchen wastes, banana peel (SSF)	Aullybux and Puchooa (2013).
*A.awamori*MTCC 9997	Brans of wheat, maize, corn, millet, rice, green gram& black gram, cassava peel powder, oil cakes of cotton seed, coconut, sesame & ground nut. (SSF)	Kalaiarasi and Parvatham (2015).
B. amyloliquefaciens	Wheat bran, soyabean meal and whey (*synergistic influence)* (semi SSF)	Yaras*et al.* (2015)
B. amyloliquefaciens	Wheat bran & straw, bran, straw & straw of rice, broken rice, maize starch, potato wastes, corncobs. (SmF)	Abd-Elhalem*et al.* (2015).
A. niger	Jackfruit, coffee husk, cassava peels (SSF)	Mathew *et al.*(2016)
*B. subtilis*PS03	What bran, paddy bran, whey wastewater, sugarcane bagasse (SmF)	Jagadeeswari and Santhi (2016).
Bacillus cereus	Wheat bran, rice straw, wheat straw, sugarcane bagasse (SmF)	Krishma andRadhathirumalaiarasu (2017).
Ascomycetes: *Bipolaris*sp., *Colleototrichum*sp., *Diparthe*sp., *Diparthe*sp., *Phomaherbarum*, &*Phyllostictacapitalensis.* Basidiomycetes:*Marasimiuscladophyllus, Phlebia*sp.&*Schizophyllum commune.*	Corncob, pineapple peels, sugarcane bagasse (SmF)	Oriandelli*et al.* (2017).
*Aspergillus*sp. SM 07	Brans of wheat, rice & maize (SSF)	Mukherjee *et al.* (2014)
Anoxybacillusamylolyticus	Potato peels, rhizomes of *Arundodonax*, *Cynaracardunculus* biomass (SmF and SSF)	Finore*et al.* (2014).
B. subtilis	Cassava peels (SmF)	Brisibe and Hankong (2014).
B. subtilis, A. niger	Sago wastes, wheat bran. (SmF)	Ruban*et al.* (2013)
A. oryzae	Rice & wheat brans, paddy husk (SSF)	Puri*et al.* (2013).
*A. oryzae*S	Soybean, wheat bran (SSF)	Chancharoonpong*et al.* (2012).
B. subtilis, B. licheniformis	Cassava starch (SmF)	Okagube and Raji (2000).
Pseudomonas fluorescens, B. subtilis, Escherichia coli, Serratiamarscens	Wheat bran, cakes of groundnut, coconut & soy (SmF)	Alariya*et al.*(2013).

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